IN THE UNITED STATED PATENT AND TRADEMARK OFFICE

Applicant: FIDIA ADVANCED BIOPOLYMERS S.r.l.

Serial No. 09/622,146

Filed on: January 2, 2001

Title: "*Sulphated hyaluronic acid and sulphated derivatives thereof covalently bound to polyurethanes, and the process for their preparation*"

DECLARATION UNDER CFR 1.132

I, Davide Renier, being sworn depose and say that:

1. I am an Italian citizen residing in Via degli Alpini 6, Mestrino (Padua), Italy
2. I am familiar with the English language.

I further declare that:

A) Education:

I have completed the bachelor degree in Industrial Chemistry in 1985.

B) Patents and publications:

I am designated inventor of four patent applications, and co-author of scientific publications. In particular, I am co-author of the communication "*Sulphation of hyaluronic acid to obtain a heparin-like molecule. Characterization and behavior in aqueous solution*" by Barbucci R., Magnani A., Lamponi, O'Regan, Renier, Pastorello in Polymers for advanced tech., Pisa, June 1995.

C) Research and professional main experiences:

I am employed as Head of Chemistry Research by Fidia Advanced Biopolymers S.r.l. As Head of Chemistry Research, I am responsible of all chemical activities and new industrial processes developed by Fidia. I have held this position since 1998. Previously, I was employed always by Fidia Advanced Biopolymers S.r.l., first as Junior Researcher (1986-1994) then as Senior Researcher in the Chemistry

Department (1994-1998). As a researcher my main activities were related to the modification of chemical structure of polysaccharides, in particular of hyaluronic acid.

3. I am familiar with the subject-matter of US Patent application No. 09/622,146 in the name of Fidia Advanced Biopolymers S.r.l., hereinafter referred to as "Fidia patent application".

4. On the basis of my personal experience and my job I declare what follows.

In the following I would like to make clear the chemistry at work in the processes disclosed by Fidia patent application and by the cited prior art patent USP No. 4,487,865, hereinafter referred to as Balazs et al.

According to Balazs et al. (see col. 2, lines 15-19), "*Sodium hyaluronate (Na-HA) is activated using the cyanogen bromide method, which has been used to attach proteins and polypeptides to cellulose or Sephadex*".

The activation method by using cyanogen bromide, referred to by Balazs et al., is in fact well known since long time. Herewith enclosed is copy of the relevant pages of the handbook "Carbohydrate Chemistry" by Kennedy J.F. (1988), wherein the mechanism of activation of polysaccharide using cyanogen bromide is illustrated. In particular, on page 570, paragraph "Activation using cyanogen bromide" of this handbook, it is clearly stated that "*Cyanogen bromide reacts with the hydroxyl groups of polysaccharides to produce derivatized matrices that react readily with primary amine groups on spacer molecules or ligands (Axen et al., 1967)*". Always on page 570, the mechanism of activation of agarose, which is a polysaccharide polymer material similar to hyaluronic acid, is schematically depicted in Figure 13.1: CN-Br reacts with an hydroxyl group –OH of agarose, forming a cyanate ester –O–C≡N, very reactive and suitable for achieving the subsequent coupling of the ligand.

Coming now to Example 3 of Balazs et al., sodium hyaluronate Na-HA is activated with cyanogen bromide CN-Br. In view of what said above, CN-Br reacts with the hydroxyl groups of Na-HA forming cyanate esters groups, which react then with the amine groups in polyurethane forming the covalent bonding reported by Balazs et al.

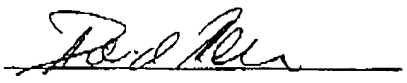
The mechanisms of the two processes disclosed by Fidia patent application are completely different. The covalent bonding occurs in both cases between polyurethane and the sulphated hyaluronic acid derivative through a spacer deriving from the two different activation agents of possible use, hexamethylenediisocyanate (HMDI) and bromoacetic acid, and in the hyaluronic acid derivative molecule the group involved in the covalent bonding is always a carboxy group –COOH, as clearly shown by the two reaction schemes reported on pages 7-10 of the Fidia patent application as originally filed.

As a matter of fact, the hydroxyl groups of the present hyaluronic acid derivatives are not involved in the bonding with polyurethane, and they could not be because the present hyaluronic acid derivatives are sulphated derivatives and the hydroxyl groups of the hyaluronic acid molecule are not free but already involved in the linking with the sulphate groups.

Therefore, the mechanisms of the Fidia processes are completely different from that of the Balazs process and, furthermore, the Balazs process could not be carried out with the present sulphated hyaluronic acid derivatives because the activation of hyaluronic acid derivative with cyanogen bromide requires the presence of free hydroxyl groups.

5. I finally declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that such willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the applications or any patents or re-examination certificate issued thereon.

Date: June 10, 2005



Davide Renier

Carbohydrate Chemistry

EDITED BY

John F. Kennedy

Research Laboratory for the Chemistry of Bioactive Carbohydrates and Proteins, Department of Chemistry, University of Birmingham, Birmingham, UK and North-East Wales Institute, Clwyd, UK

CLARENDON PRESS · OXFORD
1988

Azo bond formation

Early attempts to immobilize enzymes frequently involved use of the diazo coupling method, in which arylamino derivatives of cellulose (e.g. 4-aminobenzyl cellulose) are treated with sodium nitrite in an acidic medium (Bar-Eli and Katchalski-Katzir 1963). The reactive diazo groups thus formed couple readily with side-chains of aromatic groups (e.g. tyrosyl, histidyl) on the ligand.

Formation of peptide-like bonds

Formation of peptide-like bonds can be achieved by reaction of nucleophilic groups (e.g. amino, hydroxyl, thiol) on the ligand with suitably activated functional groups on the carrier. Although the nucleophiles are most effective in their unprotonated forms at pH's above their pK_a values, when immobilizing proteins the reactions are often performed at intermediate pH (7.5–8.5) and at low temperature (4°C) to avoid denaturation.

Activation using cyanogen bromide. Cyanogen bromide reacts with the hydroxyl groups of polysaccharides to produce derivatized matrices that react readily with primary amine groups on spacer molecules or ligands (Axén *et al.* 1967). It was at first assumed that these reactions proceeded via the cyclic imidocarbonate intermediate [13.1] shown in Fig. 13.1. However, methods for

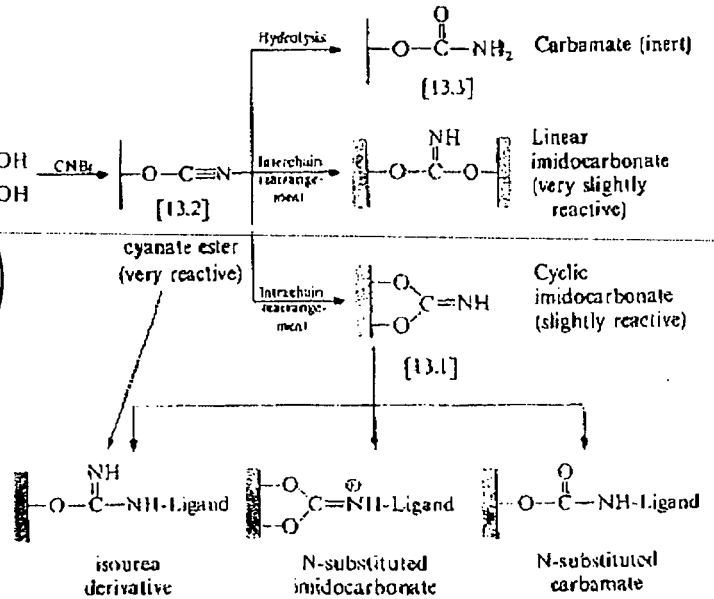
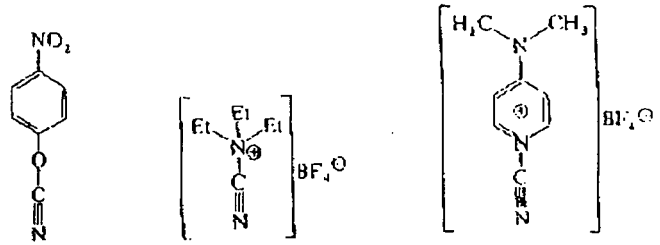


Fig. 13.1. Mechanism of activation of agarose by cyanogen bromide and subsequent

the analytical determination of cyanate esters and imidocarbonates have recently been developed, and these have allowed measurement of each of the active and inactive species produced during cyanogen bromide activation of polysaccharides (Kohn and Wilchek 1981). In freshly activated agarose 60–85 per cent of the resin's total coupling capacity was shown to be due to the formation of cyanate esters [13.2] (Fig. 13.1), imidocarbonates accounting for the remainder. Carbamates [13.3] (Fig. 13.1) formed during the activation procedures are chemically inert.

Using the information obtained from analytical determination of the reactive species the reaction mechanism shown in Fig. 13.1 has been proposed. The possibility of measuring reactive groups on the support allows prediction of coupling capacity of activated resins for ligands, and preparation of carriers containing whichever active species is required. The latter is facilitated because cyanate esters are stable below pH 4 while imidocarbonates are most stable under basic conditions. Use of triethylamine instead of sodium hydroxide in the activation procedure allows use of less than 10 per cent of the usual amount of cyanogen bromide, and the activated resins contain only active cyanate esters, with no imidocarbonates or carbamates present. Extremely high coupling capacities can be achieved.

Despite its popularity the method does suffer from several disadvantages. The *N*-substituted isourea groups formed after coupling of amines are not completely stable, particularly in the presence of nucleophiles. The resulting small leakage of ligand may lead to spurious results and, eventually, may result in some loss of the binding capacity of the matrix. In addition at physiological pH the isoureas are positively charged. This may give the matrix some anion-exchange character, resulting in increased non-specific adsorption on the support. As the cyanate ester intermediate is highly reactive, coupling should be carried out immediately after activation. The high pH (9–10) required for coupling may be unsuitable for some ligands. The high toxicity of cyanogen bromide is a practical disadvantage, although this can be avoided by using commercially available pre-activated cyanogen bromide polysaccharide matrices. Some work has been done to develop other activation reagents which react similarly to cyanogen bromide but which are more readily handled (Kohn and Wilchek 1983). These include 4-nitrophenyl cyanate [13.4], *N*-cyanotriethylammonium tetrafluoroborate [13.5], and 1-cyano-4-dimethylamino pyridinium tetrafluoroborate [13.6], all non-volatile solids



that can be stored and safely handled without use of a fume hood. These compounds have not yet replaced cyanogen bromide as major activating agents for polysaccharides, however. Despite its shortcomings the cyanogen bromide method has been widely and very successfully employed in the synthesis of many affinity adsorbents and for the immobilization of numerous enzymes.

Carbonyldiimidazole activation. Polysaccharide matrices may be activated using carbonylating agents such as *N,N'*-carbonyldiimidazole (Fig. 13.2) (Hearn *et al.* 1981). In general, the coupling achieved using this method is comparable with that of the cyanogen bromide procedure, but it has several significant advantages. The reagent is more pleasant to handle than cyanogen bromide, and the amount can be varied depending on the extent of activation required. A delay of up to six hours between activation and ligand coupling does not affect yield. Alternatively, the activated matrix can be stored in dioxane, as it is stable under anhydrous conditions. The activated carriers are available commercially. Most importantly, the carbamate (urethane) derivatives obtained on coupling with amines are stable and uncharged.

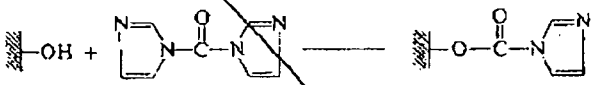


Fig. 13.2. Reaction of polysaccharide with *N, N'*-carbonyldiimidazole.

Chloroformate activation. Agarose may be activated by reaction with 4-nitrophenylchloroformate, *N*-hydroxysuccinimidochloroformate, or tri- or penta-chlorophenylchloroformate (Fig. 13.3) (Wilchek and Miron 1982). As the reaction is carried out in organic solvents, cross-linked agarose is more suitable than agarose itself. The activated carrier may be stored as a dry powder or in anhydrous organic solvents, and is commercially available. Coupling is performed in aqueous solutions. Swelling properties of the matrix in water are not much altered at low degrees of substitution but increasing substitution results in decreased swelling of the gel. Methods are available which allow the extent of activation and coupling to be measured spectrophotometrically, which is advantageous.

Reaction of cellulose or *O*-substituted celluloses with alkyl or arylchloroformates in anhydrous organic solvents yields active cellulose *trans*-2,3 carbonate derivatives which can be reacted subsequently with amino groups to form the *N*-substituted carbonate derivative (Drobnik *et al.* 1982).

Carbodiimide-promoted condensation reactions. Activation of supports containing carboxyl groups is most frequently effected using water-soluble carbodiimides [13.7] and similar reagents such as Woodward's reagent K. Under mild conditions only the more accessible or reactive carboxyl groups react, although in the presence of denaturants almost quantitative substitution can be achieved. Carbodiimide-catalyzed amide formation (Fig. 13.4) appears

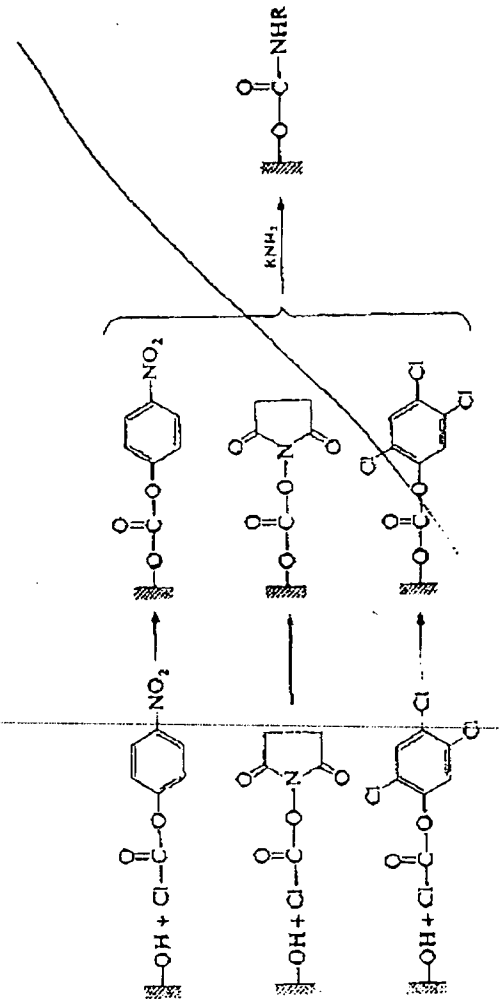


Fig. 13.3. Reaction of agarose with (a) 4-nitrophenylchloroformate; (b) *N*-hydroxysuccinimidochloroformate; and (c) trichlorophenylchloroformate.

be used to elute biospecifically bound protein while much higher concentrations of cofactor or salt may be required to elute less specifically bound proteins.

Other dyes have also been used for affinity chromatography purifications, notably the Procion® dyes. They too can be immobilized without difficulty on polysaccharide supports, and they have been applied successfully to numerous purifications. Choice of the most suitable immobilized dye for a particular application is probably best made by carrying out small-scale trial experiments, as has been well-described (Lowe and Pearson 1984).

13.4.8 Immobilization of pharmaceuticals

Antibiotics such as ampicillin, cephalexin, methotrexate, and vancomycin have been immobilized on agarose, generally for use in affinity chromatography of drug-binding proteins. The immobilization method selected depends on the chemical nature of the drug to be immobilized, but coupling is usually effected through spacer ligands following similar procedures to those adopted for cofactor immobilization.

13.4.9 Ligands for hydrophobic interaction chromatography

The presence of hydrophobic sites exposed on the surface of proteins can be exploited for purification of both water-soluble and lipophilic proteins, using hydrophobic interaction chromatography. The differing degrees of hydrophobic interaction of constituents of a mixture with an uncharged matrix containing hydrophobic groups provide a means of selectively desorbing the desired protein from the matrix. Generally the interaction of the required protein with a homologous series of alkyl-agaroses is examined, often using commercially available 'kits' for initial assessment. Each member in each series will retain only some proteins out of the mixture. When the most suitable immobilized derivative has been selected, further resolution can be achieved by gradually changing the nature of the eluent.

Alkyl–agarose derivatives which can be used for hydrophobic chromatography are shown in Fig. 13.16. The derivatives are generally prepared either by reaction of the appropriate alkyl derivative with cyanogen bromide-activated agarose (Shaltiel 1974) or by synthesis of glycidyl ethers of the required alkyl group (Ulbrich *et al.* 1964) followed by coupling of the ether to agarose (Hjertén *et al.* 1974). The use of hydrophobic chromatography as a valuable additional tool for the resolution of proteins was extensively discussed by Shaltiel (1984).

13.5 Conclusion

In this chapter space has permitted only a brief résumé of some of the most important methods for preparation of polysaccharide derivatives for use as

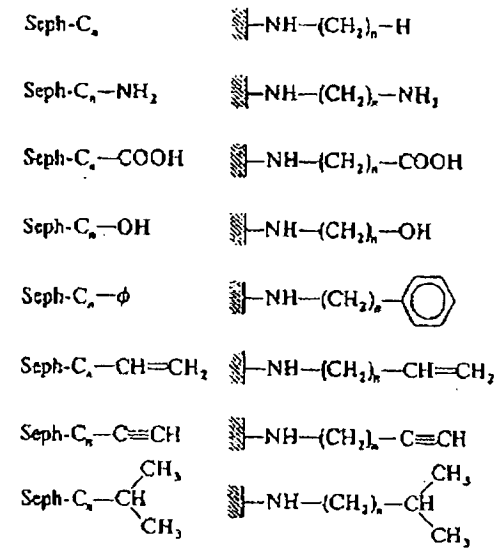


Fig. 13.16. A variety of homologous series of alkylagarose derivatives which can be used for chromatography (Shaltiel 1984).

immobilized enzymes and in affinity chromatography. Numerous other procedures have been used, and are well-documented in the scientific literature. More detailed information is provided in the excellent articles in *Methods in Enzymology* (Vol. 34, 1974; Vol 104, 1984) and in *Solid phase biochemistry* (Scouten 1983). Recent progress in affinity chromatography is also highlighted in the proceedings of the International Symposia on Affinity Chromatography (Chaiken *et al.* 1983; Gribnan *et al.* 1982). The application of these polysaccharide derivatives, which are of considerable importance in biotechnology, is discussed in Chapter 15.

References

Axén, R., Porath, J., and Ernback, S. (1967). *Nature, Lond.* **214**, 1302.
Bar-Eli, A. and Katchalski-Katzir, E. (1963). *J. Biol. Chem.* **238**, 1690.
Barker, R., Chiang, C.-K., Trayer, I. P., and Hill, R. L. (1974). *Methods Enzymol.* **34**, 317.
Birnbaum, S., Larsson, P.-O., and Mosbach, K. (1983). In *Solid phase biochemistry* (ed. W. H. Scouten), p. 679. John Wiley, New York.
Brocklehurst, K., Carlsson, J., Kierstan, M. P. J., and Crook, E. M. (1974). *Methods Enzymol.* **34**, 531.
Chaiken, I. M., Wilchek, M., and Parikh, I. (Eds.) (1983). *Affinity chromatography and biological recognition.* Academic Press, New York.
Drobnik, J., Labsky, J., Kudlvasrove, H., Sandek, O., and Svec, F. (1982). *Biotechnol. Bioeng.* **254**, 487.